Romidepsin and azacitidine synergize in their epigenetic modulatory effects to induce apoptosis in CTCL

Rozati, Sima ; Cheng, Phil F ; Widmer, Daniel S ; Fujii, Kazuyasu ; Levesque, Mitchell Paul ; Dummer, Reinhard

Abstract: Purpose Cutaneous T cell lymphomas (CTCL) are a heterogeneous group of malignancies that despite available therapies commonly relapse. The emergences of combination epigenetic therapies in other hematologic malignancies have made investigation of such combinations in CTCL a priority. Here, we explore the synergistic anti-proliferative effects of romidepsin, an HDAC inhibitor, and azacitidine, a demethylating agent, combination in CTCL. Experimental Design The growth inhibition under combination treatment and single agent was explored by the MTT cell viability assay and the Annexin V/ Propidium Iodide apoptosis assay in different CTCL cell lines and tumor cells derived from Sézary syndrome patients. Quantitative analysis of dose-effect relationship of romidepsin and azacitidine was done by the CompuSyn software. Investigation of mechanism of action was performed by flow cytometry, immunoblotting, qRT-PCR arrays and chromatin immunoprecipitation. Global CpG methylation-sequencing was utilized to study genome methylation alteration under the treatment modalities. Results The combination of romidepsin and azacitidine exerts synergistic anti-proliferative effects and induction of apoptosis involving activation of the caspase cascade in CTCL. We identified genes that were selectively induced by the combination treatment, such the tumor suppressor gene RhoB that is linked to enhanced histone acetylation at its promoter region in parallel with pronounced expression of p21. Global CpG methylation-sequencing in a CTCL cell line and tumor cells demonstrated a subset of genes with a unique change in methylation profile in the combination treatment. Conclusions The synergistic anti-proliferative effects of romidepsin and azacitidine combination treatment justify further exploration in clinical trials for advanced CTCL.

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Romidepsin and Azacitidine Synergize in their Epigenetic Modulatory Effects to Induce Apoptosis in CTCL

Sima Rozati, Phil F. Cheng, Daniel S. Widmer, Kazuyasu Fujii, Mitchell P. Levesque, and Reinhard Dummer

Abstract

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Experimental Design: The growth inhibition under combination treatment and single agent was explored by the MTT cell viability assay and the Annexin V/propidium iodide (PI) apoptosis assay in different CTCL cell lines and tumor cells derived from Sézary syndrome patients. Quantitative analysis of a dose–effect relationship of romidepsin and azacitidine was done by the CompuSyn software. Investigation of mechanism of action was performed by flow cytometry, immunoblotting, qRT-PCR arrays, and chromatin immunoprecipitation. Global CpG methylation sequencing was utilized to study genome methylation alteration under the treatment modalities.

Results: The combination of romidepsin and azacitidine exerts synergistic antiproliferative effects and induction of apoptosis involving activation of the caspase cascade in CTCL cell lines and tumor cells derived from Sézary syndrome patients. We identified genes that were selectively induced by the combination treatment, such the tumor suppressor gene RhoB that is linked to enhanced histone acetylation at its promoter region in parallel with pronounced expression of p21. Global CpG methylation sequencing in a CTCL cell line and tumor cells demonstrated a subset of genes with a unique change in methylation profile in the combination treatment.

Conclusions: The synergistic antiproliferative effects of romidepsin and azacitidine combination treatment justify further exploration in clinical trials for advanced CTCL. *Clin Cancer Res; 1–12. ©2015 AACR.*

Introduction

Cutaneous T-cell lymphomas (CTCL) comprise a heterogeneous group of malignancies derived from skin-homing T cells. The more common subtypes are mycosis fungoides, and Sézary syndrome, an aggressive leukemic variant of CTCL (1). These two subtypes account for approximately 70% to 75% of all cases (2). Therapeutic options include skin-directed therapy and/or systemic therapy (3). The success rate of most available therapy regimens ranges from 30% to 50%, but relapse is common and difficult to treat, and curative therapy remains elusive. Therefore, there is an indisputable need for more successful treatment approaches for advanced CTCL.

Romidepsin (F228 or depsipeptide) is a potent bicyclic histone deacetylase (HDAC) inhibitor approved by the FDA for the treatment of relapsed/refractory CTCL patients on the basis of two phase II clinical trials showing an overall response rate of 34% to 35% (4).

Azacitidine (5-azacitidine or Vidaza) is a cytotoxic cytidine analogue and DNA methyltransferase inhibitor with antineoplastic activity (5). At lower concentrations, it is known to relieve the transcriptional repression of various tumor suppressor genes that were silenced via hypermethylation of their promoter region (6). It was approved by the FDA for the treatment of refractory myelodysplastic syndromes (7).

In cancer, tumor suppressors are often downregulated by aberrant histone deacetylation and/or DNA methylation (8). HDAC inhibitors can synergize with demethylating agents to relieve this transcriptional repression (9). Moreover, combined epigenetic therapies have been shown to be effective in hematologic malignancies (10–13).

Here, we demonstrate that the combination of romidepsin and azacitidine exerts synergistic antiproliferative effects by inducing apoptosis in CTCL cell lines as well as $CD4^+$ T cells derived from Sézary syndrome patients with high tumor burden. We show the tumor suppressor gene RhoB as a potential regulator of the synergism, whose antineoplastic relevance has been described in other malignancies (14–16). Furthermore, our data suggest that the combination treatment results in a unique global methylation pattern in CTCL, which may contribute to increased efficacy of the antiproliferative effects of the combination treatment in CTCL.

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Translational Relevance

Potential synergistic effects between two important epigenetic regulators are under investigation in phase I/II clinical trials in refractory hematologic malignancies as well as solid tumors. Nevertheless, studies exploring the potential synergistic interactions and insight to the molecular mechanism of such combination therapy is lacking in CTCL. In addition, there has been no common functional mutation or genetic aberration identified to be responsible for malignant transformation of T cells in CTCL. In this study, we describe, the synergistic effect of romidepsin and azacitidine in CTCL cell lines and tumor cells derived from Sézary syndrome patients as well as demonstrate the potential underlying mechanisms and global methylation profile alterations effecting gene expression with potential predictive value in CTCL. Finally, the lower concentration of each drug administered in the combination as compared with single agent might translate in a favorable tolerability of this combination that will be explored in future clinical trials.

Materials and Methods

Cell lines

MyLa, SeAx, and Hut78, well-established CTCL cell lines (17, 18), were cultured in RPMI1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS, glutamine (2 mmol/L), and streptomycin (100 μg/mL) at 37°C, 5% CO_2, and 95% humidity.

Cell viability assay

The MTT (Sigma-Aldrich) assay measures the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble form, formazan, which under defined conditions can reflect the number of viable cells. This assay was utilized to measure cell proliferation in untreated and treated CTCL cell lines, Sézary syndrome patients, and healthy donor PBL samples. The absorbance was measured by spectrophotometry, using a 550 nm wavelength ELISA reader.

Flow cytometry

FITC Annexin V Apoptosis Detection Kit II (BD Biosciences) was utilized for flow cytometry to determine the percentage of cells that are actively undergoing apoptosis within the untreated and treated CTCL cell lines, Sézary syndrome patients, and healthy donor PBL samples. After designated treatment schedule, cells were harvested and stained as instructed in manufacturer's protocol.

Propidium iodide (PI; Sigma-Aldrich) was utilized as a DNA stain for flow cytometry. DNA content in cell-cycle analysis was evaluated to differentiate necrotic, apoptotic, and normal cells in the above described populations. The proportion of cells that exhibited sub-G_1 phase of the cell cycle indicates DNA degradation and apoptotic cell death. After the designated treatment time, cells were harvested in 0.3 mL of cold PBS and then fixed with cold 70% ethanol, left incubating on ice for 1 hour, and washed twice with cold PBS. RNase A (10 mg/mL) was added to each sample and incubated at 37°C for 1 hour. Cells were acquired using a BD FACSCanto flow cytometry using the FL2-A channel and analyzed with the FlowJo software 8.5.2.

Western blot analysis

Cells were washed twice with cold PBS and lysed at 4°C in RIPA protein lysis buffer. The protein concentration of each sample was determined by Bradford assay (Bio-Rad protein assay, Bio-Rad). Proteins were separated by SDS-PAGE using the NuPAGE SDS-PAGE Gel System on 4%–12% or 10% NuPage Bis-Tris gels (Life Technologies) under reducing conditions according to the manufacturer's instructions and transferred onto nitrocellulose membranes (Invitrogen). The following primary antibodies were used: anti-acetylated H3 (#9649), anti-cleaved caspase-3 (#9664, rabbit monoclonal), anti-cleaved caspase-9 (#7237, rabbit monoclonal, anti-cleaved PARP (#5625, rabbit monoclonal), $p21^{waf1/Cip1}$ (#2946, mouse monoclonal), and anti-α-tubulin (#2125, rabbit monoclonal; Cell Signaling Technology). Bound antibodies were detected using ECL Western Blotting Detection Reagent (GE Healthcare).

Immunohistochemistry

After incubation of CTCL cell lines with romidepsin and azacitidine simultaneously for 48 hours, the samples were placed on the slides using the cytospin procedure, as previously published (19). After fixation in 10% acetone, the slides were incubated with the RhoB primary antibody (C-5, mouse monoclonal, Santa Cruz Biotechnology), and then secondary antibody as suggested by manufacturer's protocol.

Primary Sézary cells and healthy donor $CD4^+$ T cells

Sample collection and laboratory studies were in compliance with Institutional review board, ethics committee and conducted according to the Declaration of Helsinki Principles. All patients signed informed consent, approved by the USZ ethical committee (EK647).

Patients' and healthy donors' peripheral blood was collected in EDTA tubes. PBMCs were separated from whole blood by Ficoll density gradient, then $CD4^+$ T cells were selected by MACS $CD4^+$ negative selection kit (Miltenyi Biotec) per the manufacturer's protocol. After negative cell selection, $CD4^+$ T cells were stimulated with CD3/CD28 Dynabeads (Life Technologies) in addition to IL2 (30 U/mL) and treated with/without romidepsin and/or azacitidine as described.

Quantitative real-time reverse-transcriptase-PCR

Total RNA was extracted after incubation with designated time of treatment or untreated cells using TRIzol according to the manufacturer's instructions (Invitrogen). Concentration was determined by NanoDrop spectrophotometer. A total of 2 mg RNA was used for cDNA synthesis using Promega's Reverse Transcription System (Promega) according to the supplied protocols. Gene expression was quantified using the Universal SYBR Green Master (ROX; 04913914001; Roche) and the Viia7 system from Applied Biosystems. The primers for were purchased from Qiagen and Cell Signaling Technology. The qRT-PCR arrays (RT^2 Profiler Cancer Drug Targets PCR Array) were purchased from SA Biosciences (Qiagen). Total RNA from untreated and treated samples was characterized in technical triplicates, and the relative expression levels for each gene in single agent or combination treated

samples were plotted against DMSO in the Scatter plot. All analyses were conducted in relative quantitation using the $\Delta\Delta C_t$-based method for calculation of relative quantitation values (20).

Chromatin immunoprecipitation-PCR

DNA was immunoprecipitated with the MAGnify Chromatin Immunoprecipitation System (Life Technologies) as per the manufacturer's instructions. Selective enrichment was performed

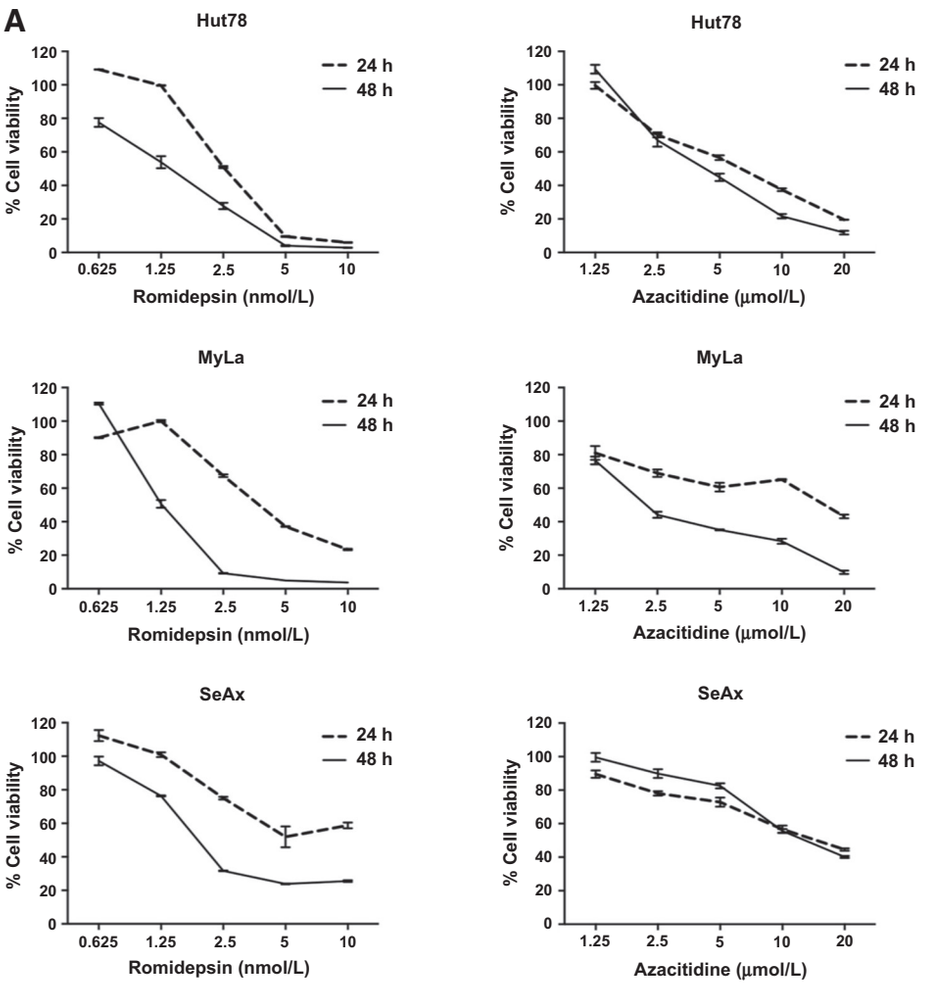
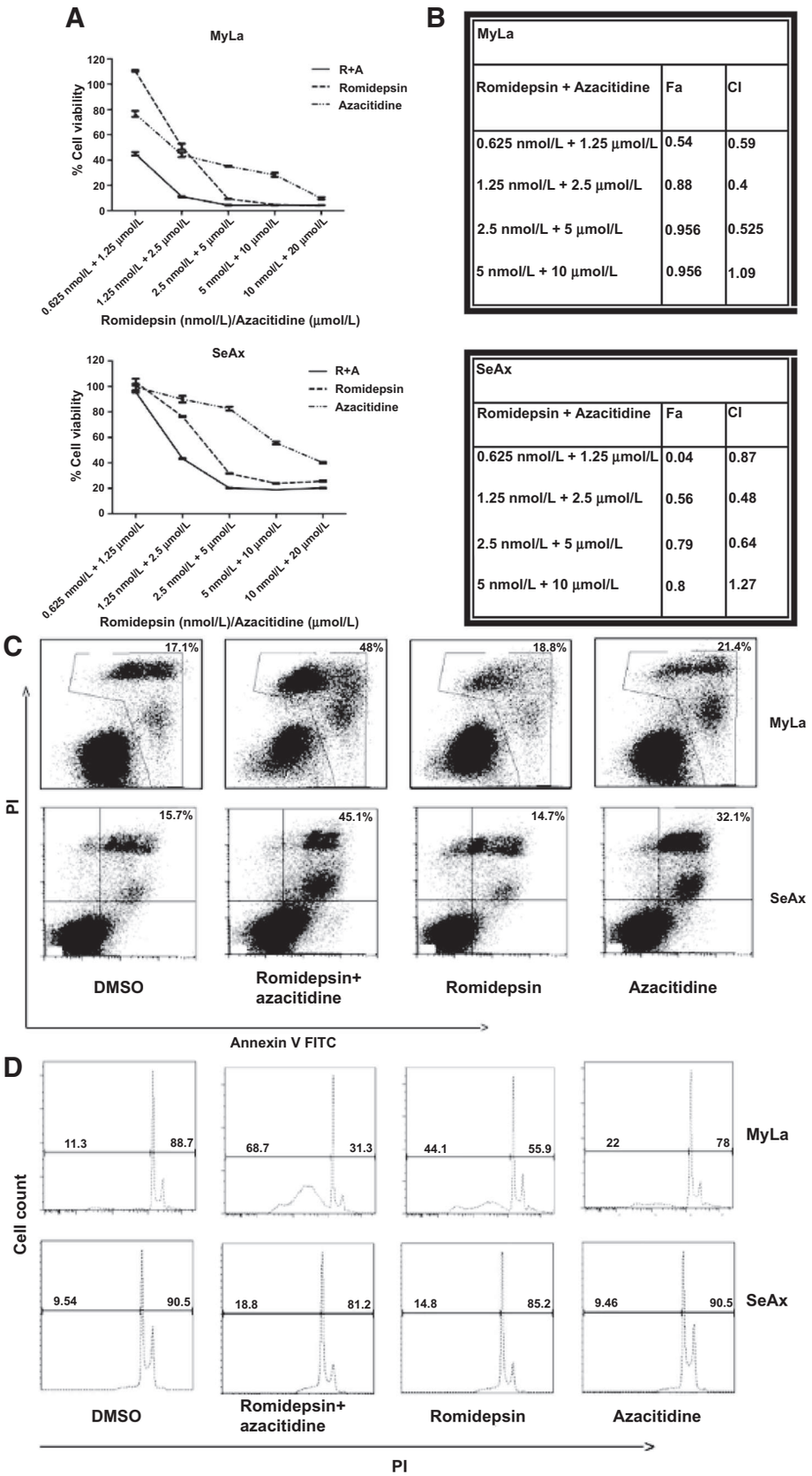


Figure 1.
Change in cell viability measured via MTT colorimetric assay. A, dose–response curves were explored for each CTCL cell line (Hut78, MyLa, and SeAx,) treated with romidepsin or azacitidine for 24 and 48 hours. Values are defined as means converted to percentages after normalization to untreated control; error bars represent SD. B, median dose effect (analogue of IC_{50}) was calculated for romidepsin and azacitidine at 24 and 48 hours for each CTCL cell line (HUT78, MyLa, SeAx).

B

		Cell line		
Drug	Time	Hut78	MyLa	SeAx
Romidepsin (nmol/L)	24 h	1.8	4.6	7.8
	48 h	1.1	2.4	3.7
Azacitidine (μmol/L)	24 h	8.4	15.8	12.6
	48 h	6.6	3.0	12.4

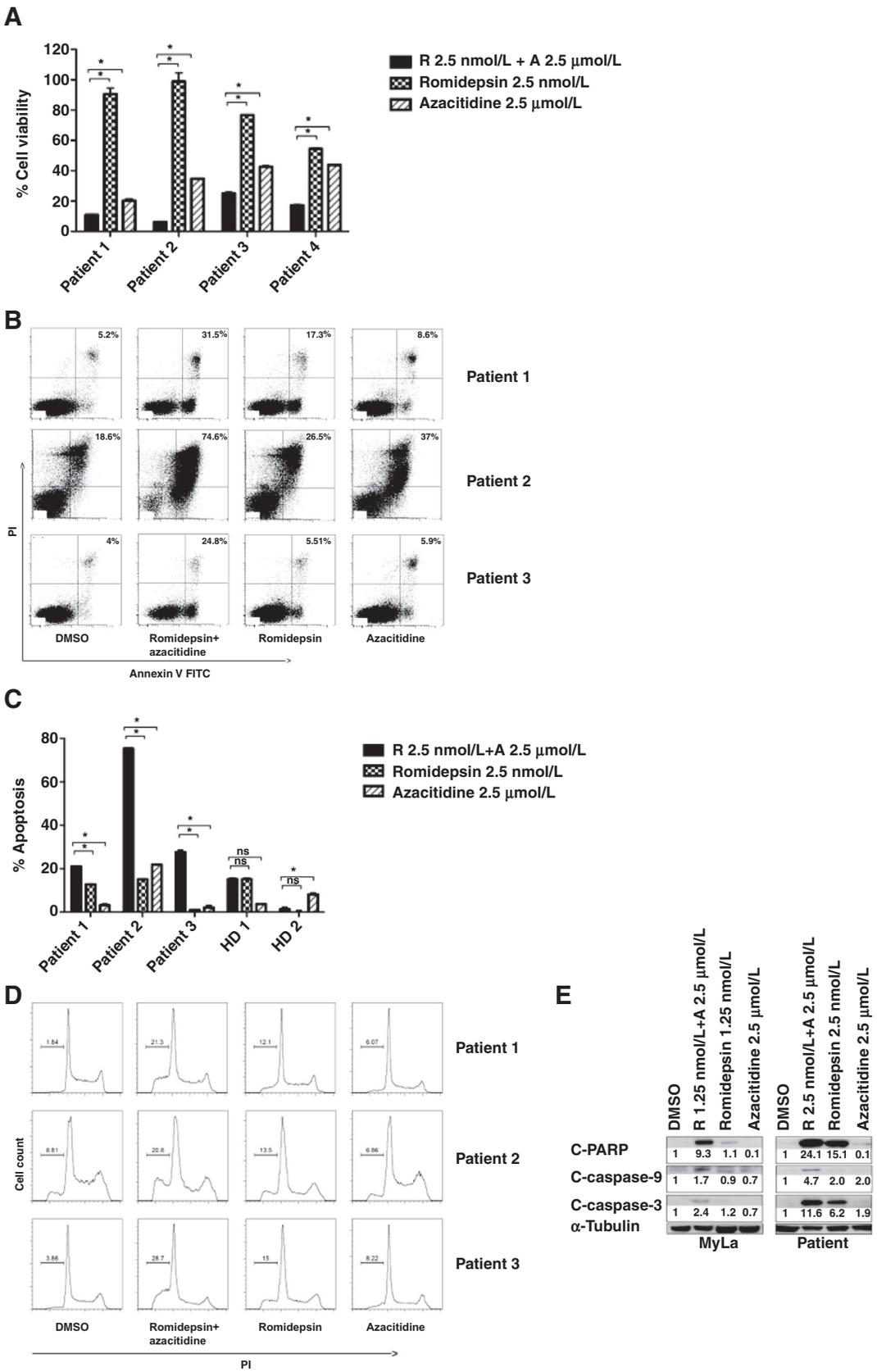


B

MyLa		
Romidepsin + Azacitidine	Fa	CI
0.625 nmol/L + 1.25 μmol/L	0.54	0.59
1.25 nmol/L + 2.5 μmol/L	0.88	0.4
2.5 nmol/L + 5 μmol/L	0.956	0.525
5 nmol/L + 10 μmol/L	0.956	1.09

SeAx		
Romidepsin + Azacitidine	Fa	CI
0.625 nmol/L + 1.25 μmol/L	0.04	0.87
1.25 nmol/L + 2.5 μmol/L	0.56	0.48
2.5 nmol/L + 5 μmol/L	0.79	0.64
5 nmol/L + 10 μmol/L	0.8	1.27

Figure 2.
Synergistic decrease in cell viability and induction of apoptosis. A, constant ratio analysis of combination treatment demonstrated synergistic decrease in cell viability as measured by MTT colometric assay after 48 hours incubation of MyLa and SeAx cell lines with romidepsin (R) and/or azacitidine (A) combination at different concentration at a constant dilution ratio of 1:2,000. B, fraction-affected (Fa) and CI are explored after 48-hour incubation with romidepsin and azacitidine combination, CI<1 represents synergy. C, MyLa and SeAx cells were treated for 48 hours with romidepsin (1.25 nmol/L) and/or azacitidine (2.5 μmol/L) or DMSO-only treated cells. Then, induction of apoptosis was measured by flow cytometry after FITC Annexin V/PI staining (values in the right top quadrants represent the percentages of cells that were Annexin V^+/PI^- plus Annexin V^+/PI^+). D, cell-cycle analysis, sub-G_1 population measured by flow cytometry after PI staining.


A
% Cell viability
120
100
80
60
40
20
0
Patient 1
Patient 2
Patient 3
Patient 4
R 2.5 nmol/L + A 2.5 μmol/L
Romidepsin 2.5 nmol/L
Azacitidine 2.5 μmol/L
B
5.2%
31.5%
17.3%
8.6%
Patient 1
18.6%
74.6%
26.5%
37%
Patient 2
4%
24.8%
5.51%
5.9%
Patient 3
PI
DMSO
Romidepsin+ azacitidine
Romidepsin
Azacitidine
Annexin V FITC
C
% Apoptosis
80
60
40
20
0
Patient 1
Patient 2
Patient 3
HD 1
HD 2
ns
R 2.5 nmol/L+A 2.5 μmol/L
Romidepsin 2.5 nmol/L
Azacitidine 2.5 μmol/L
D
Cell count
Patient 1
Patient 2
Patient 3
DMSO
Romidepsin+ azacitidine
Romidepsin
Azacitidine
PI
E
DMSO
R 1.25 nmol/L+A 2.5 μmol/L
Romidepsin 1.25 nmol/L
Azacitidine 2.5 μmol/L
DMSO
R 2.5 nmol/L+A 2.5 μmol/L
Romidepsin 2.5 nmol/L
Azacitidine 2.5 μmol/L
C-PARP
1 9.3 1.1 0.1
1 24.1 15.1 0.1
C-caspase-9
1 1.7 0.9 0.7
1 4.7 2.0 2.0
C-caspase-3
1 2.4 1.2 0.7
1 11.6 6.2 1.9
α-Tubulin
MyLa
Patient

using an antibody specific to histone H3 acetylated at K9 (Cell Signaling Technology) and unspecific IgG (Cell Signaling Technology) as control. Then, we quantified the ratio of immunoprecipitated DNA over 10% input with primers specific to the RhoB promoter region (forward primer: GGTTTCCCATTGGACGGCTA; reverse primer: GCCTCGCTGAGCATACAAGA) by qRT-PCR, in the MyLa cell line.

Next-generation DNA methylation sequencing

DNA was isolated using TRIzol, according to manufacturer's instructions. Total of 3 μg of DNA was used as input amount in the Agilent SureSelect^{XT}Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing protocol. Quality of the isolated DNA was assessed by an Agilent 2200 Tapestation. DNA was sheared using a S220 Focused-ultrasonicator (Covaris). Bisulfite conversion was done using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions.

Sequencing was performed by Illumina Hiseq 2000. We sequenced 0.25 lane per sample paired end 100 bp. Average coverage was 20×. Data analysis was done using "Bismark" for aligning and "Methylkit" for differential methylation quantification. *P* values were adjusted for multiple hypotheses testing according to the false discovery rate method. Data have been uploaded to NCBI GEO GSE66732.

Compounds and reagents

Romidepsin and azacitidine were provided by Celgene.

Statistical analysis

The median dose effect [ref. 21; or half the maximal inhibitory concentration (IC_{50})] values for each cell line at different time points were determined using CompuSyn software (22) based on the quantitative analysis of dose–effect relationships on multiple drugs or enzyme inhibitors by Chou and Talalay (23). Combinational index (CI) values were calculated to confirm synergy. CI <1 indicates synergistic effects, CI = 1 indicates the mean additive effect of the drugs, and CI > 1 represents an antagonistic effect.

Statistically significant differences within different experiments were determined using Student *t* test with $P < 0.05$ as minimal significance.

Results

Romidepsin and azacitidine combination treatment synergize in the induction of apoptosis in CTCL

We observed a concentration- and time-dependent decrease in cell viability as measured by the MTT colormetric assay, in all cell lines at 24 and 48 hours after incubation with a range of romidepsin or azacitidine concentrations (Fig. 1A). We determined the IC_{50} for romidepsin and azacitidine in all cell lines (MyLa, SeAx, and Hut78) at 24 and 48-hour time point utilizing the CompuSyn software. IC_{50} of romidepsin was within the range of what was previously reported in preclinical studies (24). The calculated IC_{50} of azacitidine varied between cell lines but remained mostly >5 μmol/L even after 48 hours of treatment (Fig. 1B).

Similar pattern in time- and dose-dependent decrease in cell viability was observed in all three cell lines. Therefore, we continued our experiments with MyLa and SeAx cell lines. We next analyzed the dose-dependent growth inhibition under romidepsin and azacitidine combination treatment and single agent, normalized to control, at different concentration levels with a constant dilution ratio of 1:2,000 at 48 hours via MTT assay (Fig. 2A). Moreover, the combination index (CI) was calculated using the CompuSyn software with CI<1 representing synergy (Fig. 2B), as detailed in the Materials and Methods section. The combination treatments were synergistic at different combination concentrations especially at lower concentrations. The lack of synergy at the highest concentration is likely due the fact that potent drugs, such as romidepsin, can become highly cytotoxic at high concentrations and are not an accurate representation of the combination effects on cell viability (25). The concentration of azacitidine, as a hypomethylating agent, in our experiments correlates with the concentration used in many preclinical experiments in various cell lines (26, 27).

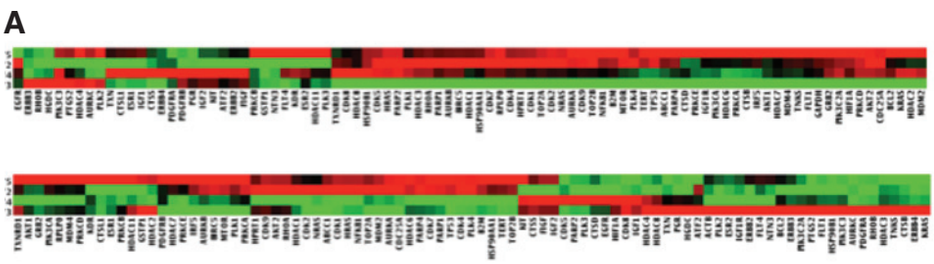
Further experiments with the CTCL cell lines were performed with romidepsin 1.25 nmol/L and/or azacitidine 2.5 μmol/L, as at these concentrations, the combination had the lowest CI reflecting a higher level of synergy at 48 hours. MyLa and SeAx cells exhibited a higher percentage of apoptosis under romidepsin 1.25 nmol/L and azacitidine 2.5 μmol/L combination treatment compared with each single agent added together as analyzed by flow cytometry after Annexin V/PI staining (Fig. 2C). Moreover, under the combination treatment, a higher percentage of MyLa and SeAx cells demonstrated a shift to the sub-G_1 population, as measured by flow cytometry after PI staining, further confirming the antiproliferative effects (Fig. 2D).

Romidepsin and azacitidine combination treatment synergize in inducing apoptosis in CD4$^+$ T cells derived from Sézary syndrome patients but not on healthy donor CD4$^+$ T cells

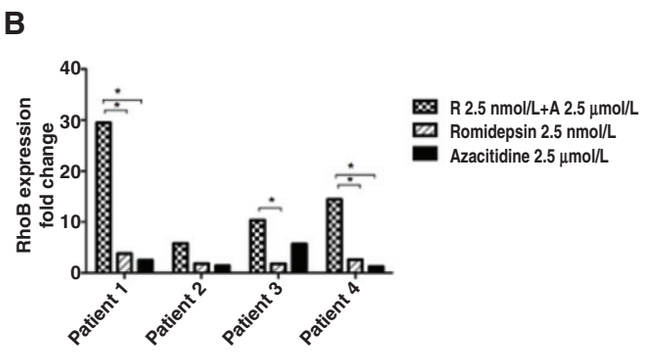
To analyze the growth-inhibitory effects of the combination treatment on Sézary cells, we separated CD4$^+$ T cells from the peripheral blood of Sézary syndrome patients and two healthy donors, as described in the Materials and Methods section. Some studies suggest that treatment with hypomethylating agents should precede treatment with HDAC inhibitors for optimum synergy (12, 28). Therefore, based on the *in vitro* experiments and preliminary experiments on tumor cells derived from Sézary syndrome patients that were treated with different concentration of romidepsin and/or azacitidine (data not shown), optimum

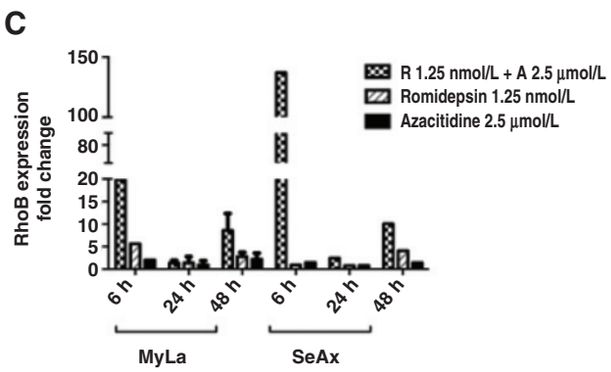
Figure 3.

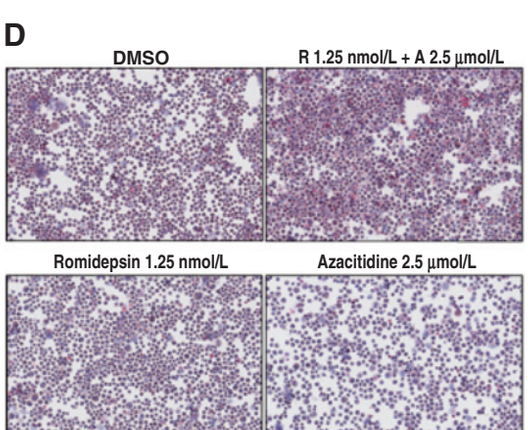
Assessment of cell viability and apoptosis in Sézary syndrome (SS) patients, healthy donor (HD) derived cells, and caspase cascade activation. A, significant decrease in cell viability in the combination treatment compared with single agents ($P < 0.05$) shown by MTT assay in 4 Sézary syndrome–derived tumor cells. B, significant increase in apoptosis in the combination treatment compared with single agents ($P < 0.05$) shown by flow cytometry after FITC Annexin V/PI staining (values in the right top quadrants represent the percentages of cells that were Annexin V$^+$/PI$^-$ plus Annexin V$^+$/PI$^+$). C, grouped graph depiction of induction of apoptosis in combination treatment versus single agent in 3 Sézary syndrome patient–derived cells compared with 2 helthy donor–derived cells. Treatments in healthy donor–derived cells only resulted in low growth inhibition without additive toxicity by the combination. D, cell-cycle analysis, sub-G_1 population measured by flow cytometry after PI staining in patient samples. E, increase in cleavage of caspase-9, caspase-3, and PARP in the combination compared to single-drug or DMSO-treated cells in MyLa cell line and representative Sézary syndrome–derived patient cells. R, romidepsin; A, azacitidine.

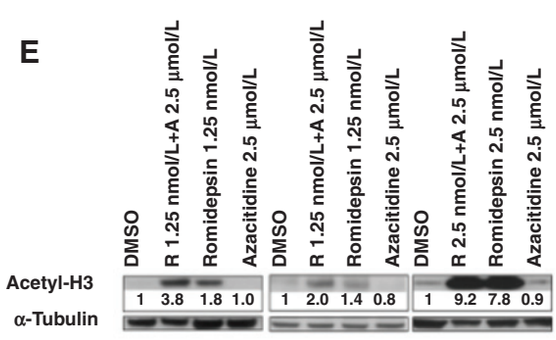


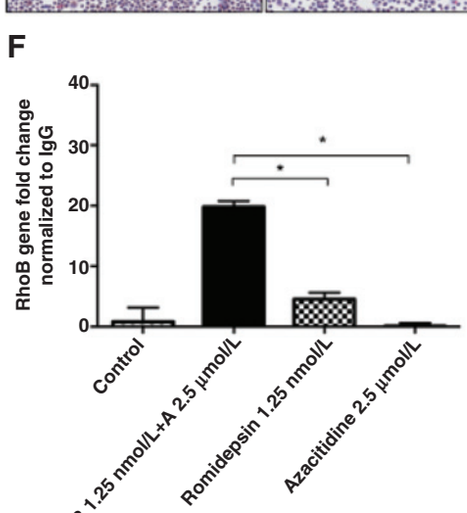
2. DMSO, 3. Romidepsin + Azacitidine, 4. Romidepsin, 5. Azacitidine

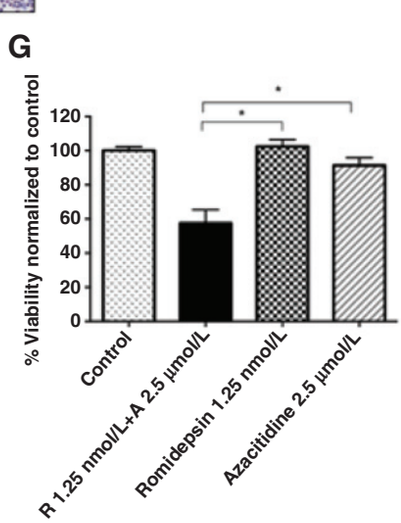


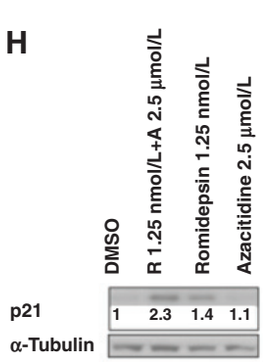












antiproliferative effects were reached with azacitidine 2.5 μmol/L for 4 days and romidepsin 2.5 nmol/L was added for the last 24 hours. This combination was used in the patient-derived samples for further experiments.

A significant synergistic effect on cell viability and induction of apoptosis was evident, measured by the MTT assay (Fig. 3A) and by flow cytometry after Annexin V/PI staining (Fig. 3B and C, $P<0.05$). To analyze the effects of this combination on $CD4^+$ cells derived from the peripheral blood of healthy donors, we incubated $CD4^+$ cells derived from healthy donors with the same schedule and noted only low cytotoxicity without increased growth inhibition under the combination treatment (Fig. 3C), indicating that the combination has synergistic growth-inhibitory effect on the tumor cells beyond increased cytotoxicity.

As demonstrated with the CTCL cell lines, a higher percentage of $CD4^+$ T cells derived from Sézary syndrome patients treated with the combination showed a shift to the sub-G_1 population, further confirming increased apoptosis (Fig. 3D). In addition, the combination treatment compared with single agent resulted in pronounced cleavage of caspase-9, caspase-3, and PARP analyzed by Western blot analysis, as shown in CTCL cell line as well as a representative patient sample (Fig. 3E).

Romidepsin and azacitidine combination treatment results in reexpression of tumor suppressor RhoB in CTCL by enhancing histone acetylation of its promoter region

To elucidate the synergistic epigenetic-modulatory effects of histone deacetylation and DNA methylation on known dysregulated cancer genes, we used the cancer drug target qRT-PCR array to analyze the CTCL cell lines (MyLa, SeAx), treated with romidepsin 1.25 nmol/L alone, azacitidine 2.5 μmol/L alone, in combination, or DMSO-treated cells for 48 hours. Analysis of this dataset demonstrated that the gene expression profile of the tumor cells treated with the combination treatment is unique in comparison with single agent, which was more pronounced in the MyLa cell line (Fig. 4A). Among top 5 genes that demonstrated more than 4-fold significant reexpression in the combination treatment compared with the single treatments and vehicle, in both cell lines, was the GTP-binding protein from the Rho protein family, RhoB. This was also the only one that was consistently differentially expressed in cell lines and in patient-derived primary cells. RhoB is suggested to be induced by genotoxic stress and mediates proapoptotic effects on transformed cells (29). The expression of RhoB is attenuated in many cancer derived cell lines compared with their normal tissue counterparts (30).

We validated the significant upregulation of RhoB in $CD4^+$ cells derived from Sézary syndrome patients upon combination treatment in most patients' samples (Fig. 4B, $P < 0.05$). Moreover, previous studies have suggested that after genotoxic stress RhoB expression is induced within in the first few hours, and then is attenuated prior to another cycle of higher expression (31). To analyze this pattern, we incubated the MyLa and SeAx cell lines with romidepsin and azacitidine and extracted RNA at 6, 24, and 48 hours and measured the fold change in RhoB gene expression. RhoB expression was rapidly upregulated under the combination treatment in both cell lines as early as 6 hours, reaching a lower level at 24 hours prior to another cycle of upregulation again at 48 hours (Fig. 4C). RhoB protein expression is illustrated by IHC in MyLa cell line (Fig. 4D).

Our methyl-sequencing data showed no methylation in the CpG island of RHOB promoter region of vehicle-treated cells. In addition, it did not demonstrate any differential methylation of RhoB promoter region under the combination treatment compared with single-agent or untreated cells (data not shown). HDAC inhibitors have been shown to reactivate RhoB expression in lung (32), ovarian (33), and thyroid cancer (34). In Fig. 4E, we demonstrated more pronounced histone H3 acetylation in CTCL cell lines and a representative patient sample under the combination treatment by Western blot analysis. We speculated that histone acetylation of the RhoB promoter region is responsible for reactivation of RhoB in CTCL and hypomethylating agent further enhances this acetylation. To test this, we performed ChIP-PCR by selective enrichment using an antibody specific to histone H3 acetylated at K9 and unspecific IgG as control, and then we quantified the ratio of immunoprecipitated DNA over input with primers specific to the RhoB promoter region by qRT-PCR, in the MyLa cell line (see Materials and Methods). Figure 4F demonstrates the significantly higher histone acetylation of the RhoB promoter region under the combination treatment compared with romidepsin alone correlating with a significant decrease in cell viability by MTT assay (Fig. 4G) and more pronounced p21 signal by Western blot analysis under the combination treatment (Fig. 4H). There was no change in histone H3 acetylation in single-agent azacitidine-treated cells.

The combination of romidepsin and azacitidine results in a specific global (CpG) methylation profile alteration

The marked activity of DNA-hypomethylating agents have been attributed to hypomethylation of key regulators of apoptosis and cell-cycle arrest (26, 35). To identify the global methylation changes under the combination treatment, MyLa cell lines and $CD4^+$ T cells derived from a Sézary syndrome patient were treated with romidepsin and azacitidine in combination or each agent alone.

A principal component analysis of the global methylation datasets demonstrated distinct clustering of azacitidine-treated tumor cells, alone and in combination, which were distant from romidepsin alone and DMSO-treated cells (Fig. 5A). The percentage of differentially methylated regions per chromosome analysis reveals a global CpG hypomethylation under azacitidine treatment, alone or in combination, but absent in romidepsin single agent–treated cells (Fig. 5B). Genome-wide CpG island methylation profiling identified 1,956 genes shared between the

Figure 4.
Combination treatment results in reexpression of the tumor suppressor gene RhoB by enhanced histone acetylation of its promoter region. A, cancer drug targets qRT-PCR array demonstrated a unique expression profile with the combination treatment in the MyLa cell line (top) and SeAx cell line (bottom) after 48 hours. B, RhoB mRNA expression was analyzed by RT-PCR in $CD4^+$ T cells derived from four Sézary syndrome (SS) patients (C) and in the CTCL cell lines at 6, 24, and 48-hour time points demonstrating the cyclic expression of RhoB. D, RhoB expression by immunohistochemistry. E, H3 acetylation after treatment with romidepsin (R) and/or azacitidine (A) or DMSO analyzed by Western blot analysis. F, H3 acetylation at the RhoB promoter region is significantly increased in the combination treatment versus single agent, analyzed by ChIP-PCR. G, parallel cell viability analysis by MTT assay. H, effect of combination therapy on p21 protein analyzed by Western blot analysis.

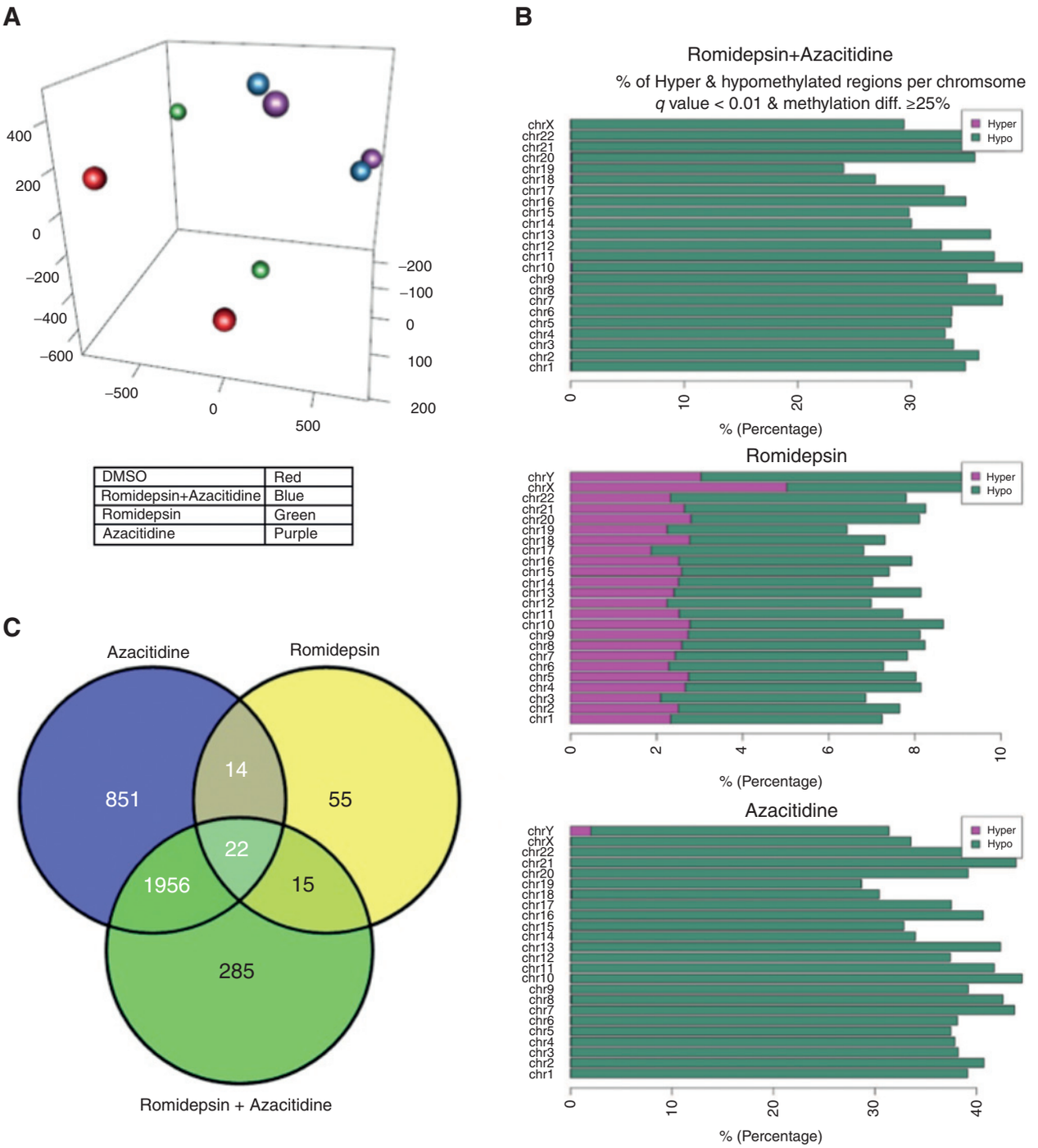


Figure 5.
Combination of romidepsin and azacitidine results in specific global methylation changes. A, principal component analysis demonstrated clustering of azacitidine-treated cells, alone or in combination away from romidepsin alone or DMSO-treated cells. B, percentage of differentially methylated regions per chromosome showed azacitidine alone or in combination causes a global CpG hypomethylation in the CTCL genome. C, a Venn diagram illustrated CpG methylation changes of overlapping genes in different treatment groups with a subset of genes that are differentially methylated only in the combination treatment (adjusted $P < 0.05$).

azacitidine and romidepsin with azacitidine treatments. A total of 285 genes were exclusive to the combination treatment (Fig. 5C). Interestingly, pathway analysis by Metacore of the genes that are differentially methylated in the azacitidine and combination treatment revealed many pathways involved in apoptosis, inflammation, and immune response.

Discussion

In many other malignancies, characteristic chromosomal abnormalities have been identified as therapeutic targets, such as bcr-abl fusion gene in chronic myelocytic leukemia (CML) and BRAF status in melanoma. In CTCL, various cytogenetic studies found multiple genetic alterations that recur in a subset of mycosis fungoides and/or Sézary syndrome patients (36–38). But few common genetic variants including targetable mutations have been identified (39).

Hence, over the past decade there has been an emergence of studies exploring the involvement of epigenetic mechanisms that contribute to the pathogenesis of CTCL (40–42). These data are limited to only molecular assessments or identification of epigenetic changes as prognostic markers.

Potential synergistic effects between two important epigenetic regulators: histone acetylation and DNA hypomethylation is under investigation in phase I/II clinical trials in refractory lymphoid and myeloid malignancies as well as solid tumors (43). Nevertheless, studies exploring the potential synergistic interactions and insight to the molecular mechanism of such combination therapy is lacking in CTCL. In this study, we describe the synergistic effect of romidepsin and azacitidine in CTCL cell lines and $CD4^+$ T cells derived from Sézary syndrome patients as well as demonstrate the potential underlying mechanisms and global methylation profile alterations affecting gene expression. Our data suggests combination of romidepsin and azacitidine in CTCL synergistically activates the caspase cascade–inducing apoptosis, as compared with single-agent treatments at the same concentrations. Moreover, the concentration of each drug administered in the combination was almost 50% less than the IC_{50} of each agent. This might translate in a favorable tolerability of this combination that will be explored in future clinical trials.

Intriguingly, the combination induced the reexpression of the tumor suppressor gene, RhoB, as early as 6 hours posttreatment. This could be explained by the rapid induction of RhoB in response to early stress to trigger pathways involved in DNA damage response and cell death. RhoB is believed to be a central factor in growth inhibition of transformed cells (15, 29, 44). Investigations in multiple cancers have found that RhoB is rarely mutated in cancer genomes (45). In addition, there is no known aberrant methylation at its promoter region that could be responsible for the loss of RhoB expression, as others (32) and we have shown. Therefore, histone modifications or other transcriptional regulatory mechanism may be responsible for the loss of RhoB expression. Preclinical data suggest increased histone acetylation at the RhoB promoter region results in its reexpression in lung carcinoma and anaplastic thyroid cancer (30, 34). In our study, the combination treatment resulted in significant histone H3 acetylation at the promoter region of RhoB gene compared with romidepsin alone suggesting histone acetylation is responsible for transcriptional activity of RhoB, which is further enhanced by the hypomethylating agent azacitidine. This might be due to the fact that DNA hypomethylation results in a more accessible chromatin for histone acetylation or due to DNA hypomethylation–independent effects of azacitidine that could contribute to the synergistic effects of the combination. It is suggested that RhoB promotes cell-cycle arrest by controlling the expression of cell-cycle regulators such as p21 (34). We observed an upregulation of p21 in cell lines and tumor cells derived from Sézary syndrome patients. This might be RhoB dependent or due to RhoB playing a crucial role in the sensitization of the CTCL cells to DNA damage and subsequent apoptosis. RhoB had a high expression under the combination treatment in the cancer drug target qRT-PCR array screen but there are other potential genes in this cancer drug target screening array that showed significant change in their expression under the combination. For example, ErbB-3 was significantly expressed under the combination treatment. This gene has been shown to play a role in sensitizing NSCLC cells to HDAC inhibitors (21). Additional detailed mechanistic experiments are required to better understand the role of these genes and their targets in CTCL.

Genome-wide methylation analysis of the MyLa cell line and tumor cells derived from an Sézary syndrome patient revealed distinct clustering of azacitidine and combination treated cells as compared with romidepsin-treated or untreated cells. This is not surprising as the majority of genes differentially methylated between azacitidine and combination treatment are shared. Romidepsin has a small effect on hypomethylation and this is in line with reports demonstrating the role of HDAC inhibition increasing global DNA hypomethylation (46, 47). Interestingly, the combination treatment had an exclusive set of differentially methylated genes, which suggests specific pathways are involved in the synergistic action of romidepsin and azacitidine. The 285 genes were involved in many signaling pathways including apoptosis, immune response, and inflammation. Overlap with the RT-PCR array for cancer target genes was not prominent, as this array is focused on mostly cancer-associated kinases. Furthermore, there could be upstream factors that when demethylated activate the genes seen in the RT-PCR array. For instance, our methylation data demonstrated SOX5 was demethylated in the combination treatment, which is known to regulate RhoB in the neural tube (48). As genes regulated by histone acetylation also contribute to the synergistic effect, genome-wide histone acetylation analysis would be necessary to fully understand the mechanisms of action of the combination treatment. Nonetheless, the differentially methylated genes specific in the combination treatment underline the important contribution of demethylation to the synergistic effect. This profile might serve as the basis for the identification of a methylation signature that is predictive of clinical response as shown in myelodysplastic syndrome (49) or to a limited extent suggested recently in CTCL (50).

In summary, there has been no common functional mutation or genetic aberration identified to be responsible for malignant transformation of T cells in CTCL. Our data provide insight to unique methylation profile alterations with potential predictive value due to the synergistic antiproliferative effects of combined romidepsin and azacitidine treatment in CTCL, and paves the road for combining these agents in clinical trials for advanced CTCL.

Disclosure of Potential Conflicts of Interest

S. Rozati has ownership interest (including patents) in patent submitted based on presented data. R. Dummer reports receiving a commercial research grant from Celgene. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rozati, P.F. Cheng, K. Fujii, M.P. Levesque

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Rozati, P.F. Cheng, D.S. Widmer, K. Fujii, M.P. Levesque, R. Dummer

Writing, review, and/or revision of the manuscript: S. Rozati, M.P. Levesque, R. Dummer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Rozati, D.S. Widmer, M.P. Levesque, R. Dummer

Study supervision: S. Rozati, M.P. Levesque, R. Dummer

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